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CHARACTERIZATION OF THE FUNCTIONAL GROUPS OF BIOTIN*

By KLAUS HOFMANN,† DONALD B. MELVILLE,‡ and VINCENT du VIGNEAUD

(From the Department of Biochemistry, Cornell University Medical College, New York City)

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In a previous publication (1) we have described a procedure for the isolation from liver of biotin as the pure crystalline methyl ester. Some of the properties of the pure substance were described, and in a later paper (2) we have given the preparation and properties of free biotin itself. We have also recently reported the results of a series of inactivation experiments on pure biotin which gave some indication of the possible presence or absence of certain types of groups (3). In this present paper we give the results of experiments which by direct chemical attack have led to the recognition of the functional groups present in the biotin molecule. We have obtained evidence that biotin is an N,N′-disubstituted cyclic urea derivative.

As previously reported (1), biotin methyl ester possesses a melting point of 166–167° and is optically active ($[\alpha]_D^{22} = +57°$ in chloroform). The analytical data are in best agreement with the empirical formula of $C_{11}H_{18}O_3N_2S$ and show one methoxyl group to be present. The free biotin (2) melts at 230–232°, possesses a rotation of $[\alpha]_D^{22} = +92°$ in 0.1 N NaOH, and is predominantly acidic in character. Analyses of the compound led to the formula $C_{10}H_{16}O_3N_2S$ which is in agreement with the results of the analyses

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‡ S.M.A. Corporation Fellow.

of the ester. Evidence for the presence of a carboxyl group in biotin has already been presented. The absence of specific absorption bands for biotin in the ultraviolet range suggests the absence of an aromatic ring or of similar structures (2).

Because biotin is inactivated by nitrous acid, it has been assumed that the compound could be an amino acid (4). We have found, however, that no nitrogen is produced when biotin is treated with nitrous acid by the Van Slyke procedure.[1] Furthermore, no color is formed after treatment of biotin with ninhydrin at pH 6.5. The possibility of biotin being an α-amino acid is consequently eliminated. Inactivation experiments likewise led to this conclusion (3).

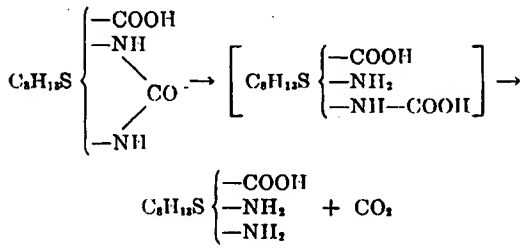
Many other attempts were made to characterize the nitrogen in the biotin molecule. It would be unfruitful, however, to give the many negative experiments in this direction, since the following observation supplied us with the information we desired as to the nature of both of the nitrogen atoms. This same finding also characterized the remaining oxygen atom to be accounted for.

Treatment of biotin or its methyl ester with strong barium hydroxide solution for 20 hours at 140° brings about the formation of a new, optically active compound which can be isolated in 85 per cent yield as the sulfate. The analyses of this compound agree best with the formula $C_9H_{20}O_6N_2S_2$. Since one-half of the sulfur in the molecule is present as ionizable sulfate, it appears that the compound is the salt of a substance possessing basic properties. The free compound, $C_9H_{18}O_2N_2S$, obtained from the sulfate by treatment with the calculated amount of barium hydroxide, melts with decomposition at 185–190°, and can be sublimed *in vacuo*.

By a micro-Van Slyke procedure an average amino N content of 8.7 per cent was observed for the sulfate of this compound. This indicates the presence of two primary amino groups in the molecule. On benzoylation by the Schotten-Baumann method an alkali-soluble dibenzoyl derivative (m.p. 182–183°) is formed which with diazomethane forms a dibenzoyl methyl ester (m.p. 128–130°). The new compound will be referred to hereafter as the diaminocarboxylic acid.

[1] We are indebted to Dr. Fritz Lipmann for carrying out the micro-Van Slyke analyses by the method of Warburg (5).

The most logical interpretation we can place on the formation of a diaminocarboxylic acid with the loss of 1 carbon atom and 1 oxygen atom from biotin is the cleavage of a cyclic urea derivative. It will be recalled that biotin is predominantly acidic, so much so that biotin crystallizes as the free compound from fairly strong acid solutions. The nitrogen atoms must therefore be extremely weakly basic. However, some basicity is indicated by the fact that the ester can be extracted from chloroform by fairly strong HCl. The conversion of the biotin possessing such weakly basic nitrogen to the much more basic diaminocarboxylic acid fits in with the interpretation offered. The inactivation of biotin by nitrous acid in spite of no liberation of nitrogen gas may possibly be due to the formation of a nitroso derivative, a property of urea derivatives. During the drastic barium hydroxide treatment the urea structure would probably be transformed by the addition of water into the corresponding carbamic acid, which then loses CO_2 to yield the diaminocarboxylic acid. Therefore, the hydrolytic cleavage of biotin may be written in the following manner.



The urea grouping must be part of a ring system, since no nitrogen and only 1 carbon atom is lost from the biotin by this procedure. The formation of two *primary* amino groups during the hydrolysis indicates further that each of the 2 nitrogen atoms of biotin carries 1 hydrogen atom.

With the characterization of the 2 nitrogen atoms and the 3 oxygen atoms, attention was focused on the nature of the sulfur. It was found that biotin does not contain alkali-labile sulfur and does not liberate H_2S when treated with zinc dust and HCl. After treatment with bromine water no inorganic sulfate could be detected. No positive nitroprusside test was obtained either in

the presence or the absence of sodium cyanide. The stability of the sulfur pointed to a thio ether structure and the experiments which will be described offer support for this assumption.

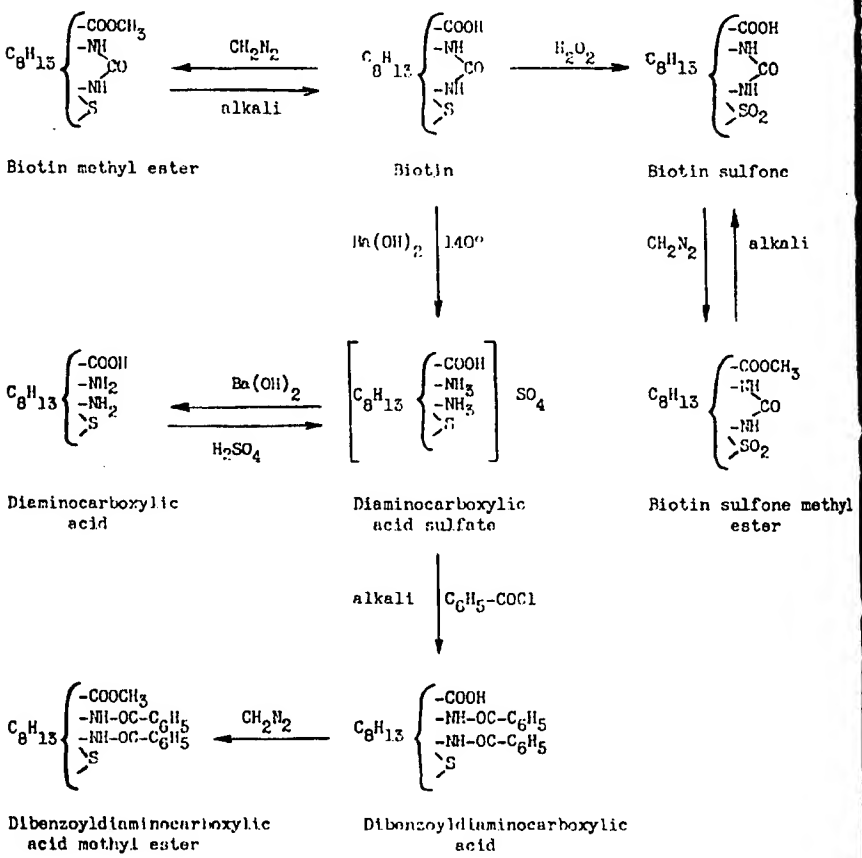


FIG. 1

It had been observed by earlier investigators, as well as by us (3), that biotin is extremely sensitive to peroxides. Accordingly, pure biotin was treated with excess hydrogen peroxide in glacial acetic acid solution at room temperature for 16 to 18 hours. From the reaction mixture it was possible to isolate a crystalline

oxidation product in 90 per cent yield. The analyses of the pure compound, which melts at 274–275° with decomposition, point to the composition $C_{10}H_{16}O_5N_2S$, in which 2 atoms of oxygen have been added to the biotin molecule without loss of carbon or hydrogen. The presence of a carboxyl group in the compound is shown by the formation of a methyl ester, m.p. 239–241°, on treatment with diazomethane. The methyl ester is saponified by cold dilute alkali with the formation of the original oxidation product, m.p. 274–275°.

Biotin and the diaminocarboxylic acid when treated with tetranitromethane produce a strong yellow color, in contrast to the new oxidation product which does not produce any color with that reagent. This behavior could parallel the formation of a sulfone from a sulfide, the latter giving strong color, the former remaining colorless when treated with tetranitromethane. Since biotin is not hydrogenated when shaken in the presence of platinum with hydrogen, the molecule apparently does not contain an ethylenic linkage, assuming no poisoning of the catalyst. The sulfur in the thio ether form would therefore seem to be responsible for the color reaction with tetranitromethane. These facts along with the addition of 2 oxygen atoms to the molecule without loss of carbon or hydrogen point to an oxidation by the peroxide treatment of a thio ether to the corresponding sulfone.

The relationships of the diaminocarboxylic acid and of the sulfone to biotin and to the derivatives of all three compounds are shown in Fig. 1.

EXPERIMENTAL

Diaminocarboxylic Acid Sulfate—10 mg. of biotin or biotin ester were heated in a sealed tube with 1 cc. of water and 200 mg. of barium hydroxide for 20 hours at 140°. The excess baryta was removed with carbon dioxide and the filtrate from the barium carbonate was acidified with 1 N H_2SO_4 until it was faintly acid to Congo red. The precipitated barium sulfate was removed by filtration and the clear filtrate was concentrated *in vacuo* to a small volume. On addition of a few drops of methanol to the solution plate-like crystals appeared. The crystals were collected by filtration and were washed with methanol. The resulting 10 mg. of crystals were further purified by crystallization from a

mixture of water and methanol. The pure material melted at 245–255°,[2] depending upon the rate of heating. It possessed a rotation of $[\alpha]_D^{22} = -15°$ for a 1 per cent solution in water.

$C_9H_{20}O_6N_2S_2$ (316.4)
Calculated. C 34.16, H 6.37, N 8.85, S 20.27, NH_2-N 8.85, SO_4-S 10.13
Found. " 34.43, " 6.31, " 8.54, " 20.22, " 8.69, " 10.07

Diaminocarboxylic Acid—10 mg. of the diaminocarboxylic acid sulfate were dissolved in 0.5 cc. of water and 0.64 cc. of 0.1 N barium hydroxide solution was added. The barium sulfate was removed by filtration and the filtrate was concentrated to dryness *in vacuo*. The crystalline residue, 5.8 mg. of needles melting at 180–185°, was purified by sublimation *in vacuo* (10^{-5} mm.) at 160°. The purified material melted at 186–190° with decomposition.

$C_9H_{18}O_2N_2S$. Calculated. C 49.52, H 8.31, NH_2-N 12.83
(218.3) Found. " 49.33, " 8.30, " 14.30

2 mg. of the sublimed material were dissolved in 0.5 cc. of water and the solution was acidified with 1 N H_2SO_4. On evaporation *in vacuo* and crystallization of the residue by the addition of methanol, the sulfate was obtained, melting at 245–250°.

Dibenzoyldiaminocarboxylic Acid—15 mg. of the diaminocarboxylic acid sulfate were dissolved in 1 cc. of water, and 1 N NaOH was added until the solution was alkaline to phenolphthalein. The solution was cooled with ice. 17 mg. of benzoyl chloride were added, and the solution was shaken for 15 minutes, and kept alkaline by the addition of 1 N NaOH. At the end of this time the alkaline solution was extracted with ether and the ether extracts were discarded. The aqueous layer was acidified to Congo red with 3 N HCl, whereupon the dibenzoyl derivative separated as an oil. The oil was extracted with chloroform. The chloroform solution was washed with water, dried over sodium sulfate, and evaporated to dryness. The crystalline residue was purified by crystallization from a mixture of methanol

[2] The melting points reported herein were determined by the use of the Kofler micro melting point apparatus and are uncorrected.

and ether. The yield of pure compound was 15 mg. of needles melting at 182–183°.

$C_{23}H_{26}O_4N_2S$ (426.5). Calculated, N 6.57; found, N 6.44

Dibenzoyldiaminocarboxylic Acid Methyl Ester—10 mg. of the dibenzoyldiaminocarboxylic acid were dissolved in 0.5 cc. of methanol and to this solution was added a freshly distilled solution of diazomethane in ether until the yellow color remained. The solution was kept in the refrigerator for 30 minutes, and was then evaporated to dryness *in vacuo*. The crystalline residue was purified by crystallization from a mixture of methanol and ether. 6 mg. of needles melting at 128–130° were obtained.

$C_{24}H_{28}O_4N_2S$. Calculated. C 65.42, H 6.40
(440.5) Found. " 65.46, " 6.36

Biotin Sulfone—11.9 mg. of biotin were dissolved in 5.4 cc. of glacial acetic acid and 0.6 cc. of 30 per cent H_2O_2 were added to the solution. The clear solution was kept at room temperature for 18 hours and then evaporated to dryness *in vacuo*. The crystalline residue was dissolved in a few drops of boiling water and the solution was allowed to cool. The biotin sulfone crystallized from the cold solution in long needles. The crystals were removed by filtration and were washed with cold water. The yield of crystals was 7.0 mg., m.p. 274–275° with decomposition. By concentration of the mother liquors an additional 5.0 mg. of the sulfone, m.p. 274–275°, were obtained.

$C_{10}H_{16}O_3N_2S$. Calculated. C 43.47, H 5.84, N 10.13, S 11.61
(276.3) Found. " 43.36, " 5.76, " 10.17, " 11.32

The biotin sulfone was esterified in the following manner. 5.0 mg. were suspended in 2 cc. of methanol and the suspension was cooled in an ice bath and treated with an excess of diazomethane for ½ hour with frequent shaking. At the end of this time the sulfone had dissolved and the yellow color due to the diazomethane remained. The solution was concentrated to dryness *in vacuo* and the crystalline residue, m.p. 238–240°, was sublimed at 220° and 10^{-5} mm. pressure. The sublimate was dissolved in 1 cc. of hot methanol and upon the addition of ether the substance crystallized from the solution. The crystals were

washed with ether and dried. The yield of biotin sulfone methyl ester, m.p. 239–241°, was 4.5 mg. The methoxyl determination indicated the presence of one methoxyl grouping.

Approximately 1 mg. of the biotin sulfone methyl ester was dissolved in a few drops of 2 N NaOH and the solution was acidified with dilute HCl. The crystalline material which separated was washed with water. This material melted with decomposition at 274–275°, the melting point of biotin sulfone.

The authors wish to express their appreciation to Dr. J. R. Rachele of this laboratory for carrying out the microanalyses

SUMMARY

Evidence accounting for the functional groups of biotin has been presented. It has been concluded that biotin is a carboxylic acid containing an N,N′-substituted cyclic urea grouping and possessing sulfur in a thio ether linkage.

By alkaline treatment of biotin a sulfur-containing diaminocarboxylic acid containing 1 less carbon atom and 1 less oxygen atom is formed. The sulfone of biotin has been prepared by the oxidation of biotin with H_2O_2. The preparations of various derivatives of these compounds have likewise been presented.

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A RAPID EXTRACTOR FOR URINARY STEROIDS

II. MODIFICATIONS FOR THE SIMULTANEOUS HYDROLYSIS AND EXTRACTION OF URINE WITH ANY SOLVENT HEAVIER THAN WATER

By E. B. HERSHBERG* and JOHN K. WOLFE†

(*From the Converse Memorial Laboratory, Harvard University, Cambridge*)

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Since our publication last year (1), the extractor with a sintered glass disk dispersing carbon tetrachloride has been employed widely in both assay and research laboratories. In addition, the principle of simultaneous hydrolysis and extraction of urine which was suggested has been shown by Talbot, Butler, MacLachlan, and Jones (2) to result in a greatly improved androgen recovery. This, and other work, indicates that considerable time can be saved and a maximum yield of hormone obtained by warming the urine in the extractor with an electrical heater until the equilibrium temperature is reached. With carbon tetrachloride as the extracting solvent this is approximately 60°, and once this temperature is reached the current may be shut off.

The operating temperature can be controlled either above or below 60° by the use of some solvent other than carbon tetrachloride as the extracting medium. A new level is then required in the extractor overflow arm to compensate for the change in specific gravity of the solvent. We have done this by using the sliding cylinder and barrel assembly *B*, shown in Fig. 1. The position of the overflow hole *C* may be adjusted while the extractor is in operation by sliding the plunger *A* up or down through the cork *D* until the porous plate just dips into the urine.

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† Research Fellow on a grant from the National Cancer Institute to Professor L. F. Fieser. Present address, National Institute of Health, Bethesda, Maryland.